

# L-Methionine Reduces Oxidant Stress in Endothelial Cells: Role of Heme Oxygenase-1, Ferritin, and Nitric Oxide

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## ABSTRACT

The amino acid L-methionine is known to exert antioxidant effects by as yet unidentified mechanisms. In the present study, L-methionine led to a concentration-dependent induction of the antioxidant proteins heme oxygenase-1 (HO-1) and ferritin in cultured endothelial cells (ECV 304). HO-1 protein expression was accompanied by an increased catalytic activity of the enzyme. Long-term pre-incubation of endothelial cells with L-methionine reduced NADPH-mediated radical formation by up to 60%. The antioxidant effect of L-methionine was mimicked by the HO-1 product bilirubin, which suppressed free radical formation almost completely. Reduction of superoxide generation by L-methionine was inhibited in the presence of the nitric oxide (NO) synthase inhibitor L-NMMA, suggesting the involvement of endogenous NO in L-methionine-dependent cytoprotection. These findings demonstrate that L-methionine reduces free radical formation in endothelial cells, possibly through induction of heme oxygenase-1 and ferritin. This novel, indirect antioxidant action might be relevant for the preventive potential of methionine and methionine rich diets under conditions of inflammation and oxidative stress.

**KEYWORDS:** L-methionine, heme oxygenase-1, ferritin, micronutrients, antioxidant

## INTRODUCTION

Oxidant stress is a causative factor of endothelial dysfunction and plays an important role in the pathophysiology of several vascular diseases such as atherosclerosis, diabetes, or neurodegenerative diseases. Emerging evidence suggests an important role of nutritional factors (eg, antioxidants, fatty acids, bioactive peptides, or free amino acids) in modulating endothelial function. L-alanine and structurally similar amino acids have been reported to reduce endothelial cell death caused by reactive oxygen species.<sup>1-4</sup> Similarly, the essential amino acid L-methionine shows antioxidant properties in various models of oxidative stress. Antioxidant effects

of L-methionine lead to the reduction of lipid peroxidation, protection against membrane damage, and to restoration of changes in the glutathione system.<sup>5,6</sup> Methionine residues can act as powerful endogenous antioxidants in proteins.<sup>7-9</sup> Moreover, L-methionine has also been reported to reduce liver damage caused by lead exposure or treatment with acetaminophen.<sup>10,11</sup> The mechanisms responsible for the observed L-methionine-induced cytoprotection are not yet fully understood. The free radical scavenging activities of methionine can only partially be explained by the chelating function of its sulfur moiety. However, a plausible mode of action might be the induction of tissue protective genes and proteins that reduce damage inflicted by reactive oxygen species (ROS).

Recently, the stress proteins heme oxygenase (HO-1) and ferritin have been identified as targets and antioxidant mediators of drugs and micronutrients.<sup>3,12,13</sup> HO-1 is an inducible enzyme that catalyzes the degradation of heme. This process leads to generation of bilirubin, iron, and carbon monoxide (CO). Bilirubin exerts strong antioxidant effects at physiological plasma concentrations. High-normal plasma levels of bilirubin were reported to be inversely related to atherogenic risk and to provide protection against endothelial damage. CO has likewise been shown to produce anti-apoptotic and cytoprotective actions.<sup>14,15</sup>

The HO-1-dependent release of free iron from the core of the heme molecule results in the up-regulation of ferritin protein expression. Ferritin provides antioxidant cellular protection by rapidly sequestering free cytosolic iron, which is the crucial catalyst of oxygen-centered radical formation via the Fenton reaction in biological systems.<sup>16,17</sup> Thus, ferritin plays an important role as a fast-acting endogenous cytoprotectant in cellular antioxidant defense mechanisms.<sup>18,19</sup>

In the present study we investigated the effect of L-methionine on the expression of the antioxidant stress proteins, HO-1 and ferritin, and their contribution to the reduction of radical formation seen under the influence of this amino acid.

## MATERIALS AND METHODS

### Materials

Fetal bovine serum, cell culture media, and gentamycin were obtained from Gibco (Eggenstein, Germany). The

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Chemiluminescence Western Blotting Kit was from Amersham (Freiburg, FRG). HO-1 primary antibody was obtained from Alexis (Grünberg, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

### Cell Culture

The human endothelial cell line ECV304 was obtained from the European Collection of Cell Cultures.<sup>20</sup> ECV304 endothelial cells were maintained and subcultured in M199 medium containing 10% fetal bovine serum and gentamycin (50 µg/mL). The cells were grown in a humidified incubator at 37°C and 5% CO<sub>2</sub>.

### Formation of ROS

NADPH (nicotinamide adenine dinucleotide phosphate)-dependent ROS formation was measured by monitoring lucigenin-derived chemiluminescence at 37°C using the Berthold LB96V luminometer according to previously published protocols.<sup>21,22</sup> Cells were cultured in 12-well plates. After a pretreatment with L-methionine for 24 hours, cells were suspended in PBS and subsequently, lucigenin (50 µM) and NADPH (100 µM) were added. L-N<sup>G</sup>-Monomethyl arginine (L-NMMA) and zinc deuteroporphyrin IX 2,4-bis-ethylene glycol (ZnBG) were added 30 minutes prior to L-methionine; bilirubin was added directly to the suspended cells. Chemiluminescence was measured in relative light units (RLU) every 5 minutes over a period of 30 minutes. Data shown represent the mean of peak values of the 30-minute measurement in percent of maximal light emission (RLU<sub>max</sub> %) of NADPH-treated control cells.

### HO-1 and Ferritin Protein Analysis

Endothelial cells were cultured in 100-mm dishes as described above. After a 24-hour incubation with control media or L-methionine, cells were washed and extracted as described previously.<sup>23</sup> Protein (100 µg for HO-1 and 20 µg for ferritin protein analysis) was applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis. After electrophoresis, protein was transferred to a nitrocellulose membrane, and a polyclonal antibody to HO-1 and ferritin was used to identify the protein content. Antigen antibody complexes were visualized with the horseradish peroxidase chemiluminescence system according to the manufacturer's instructions (Amersham, Freiburg, Germany). Quantitation of the protein content was performed using computer-assisted videodensitometry (Eagle Eye II-system, Stratagene, La Jolla, CA).

### HO Activity (Bilirubin Formation)

Confluent endothelial cells in 150-mm dishes were incubated for 24 hours in the presence of control media or L-methionine. The method used for the determination of HO-1 activity

via bilirubin formation follows the protocol published by Motterlini et al.<sup>24</sup> Briefly after the incubation, cells were washed twice with phosphate-buffered saline, gently scraped off the dish, and centrifuged (5000 rpm, 10 minutes, 4°C). The cell pellet was suspended in MgCl<sub>2</sub> (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at -80°C, thawed 3 times, and finally sonicated on ice before centrifugation at 4000 rpm for 10 minutes at 4°C. The supernatant (400 µL) was added to a NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, and 2 mg protein of rat liver cytosol prepared from the 105 000-g supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 µM) in a final volume of 200 µL. The reaction was conducted for 1 hour at 37°C in the dark and terminated by addition of 800 µL chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM<sup>-1</sup> cm<sup>-1</sup> for bilirubin). HO-1 activity was measured as picomoles of bilirubin formed per milligram of endothelial cell protein per hour (data in percent).

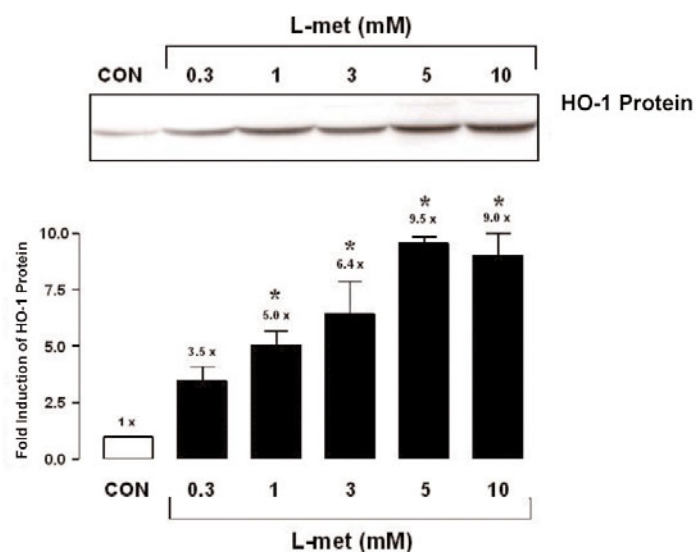
## RESULTS

### Protein Expression and HO-1 Activity

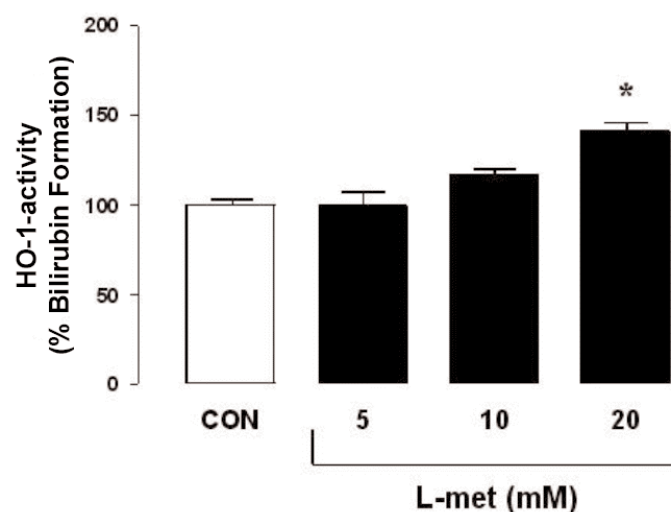
In endothelial cells, a 24-hour incubation with L-methionine produced a concentration-dependent increase in HO-1 protein levels up to 9.5-fold over basal (Figure 1). HO-1 induction was associated with a marked increase in protein expression of a secondary antioxidant protein, ferritin (Figure 2). In addition, L-methionine increased HO-1 activity, ie, formation of the antioxidant metabolite bilirubin in the cell lysate (Figure 3).

### Formation of ROS

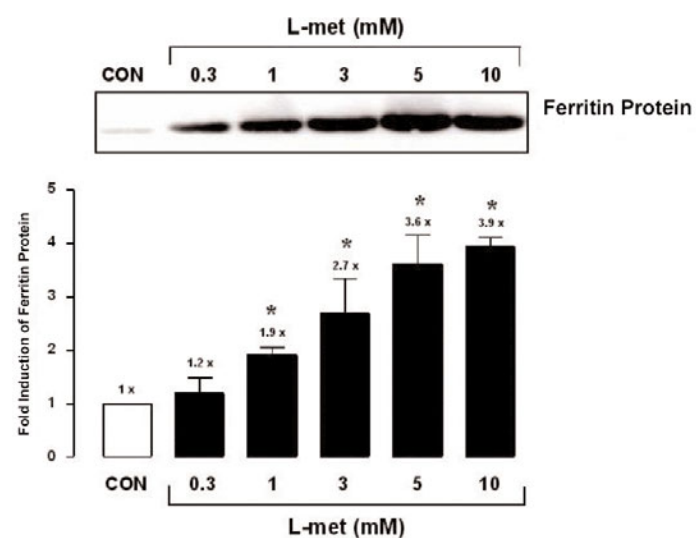
Long-term pre-incubation of endothelial cells (24 hours) with L-methionine reduced NADPH-mediated radical formation by up to 60% (Figure 4). The antioxidant effect of L-methionine was mimicked by exogenous bilirubin, which led to an almost complete suppression of free radical formation (Figure 5). In order to explore a potential involvement of HO-1 products such as bilirubin in the observed antioxidant effect of L-methionine, the HO-1 inhibitor ZnBG was used. At concentrations that were previously shown to specifically block HO activity and to not interfere with other hemoproteins,<sup>25</sup> ZnBG abrogated the reduction of NADPH-dependent O<sub>2</sub><sup>-</sup> formation by L-methionine (Figure 6). Moreover L-methionine-induced blockade of free radical formation was rescued in the presence of the nitric oxide (NO) synthase inhibitor L-NMMA (Figure 7). ZnBG or L-NMMA alone were without influence on NADPH-mediated radical formation under these conditions (not shown).



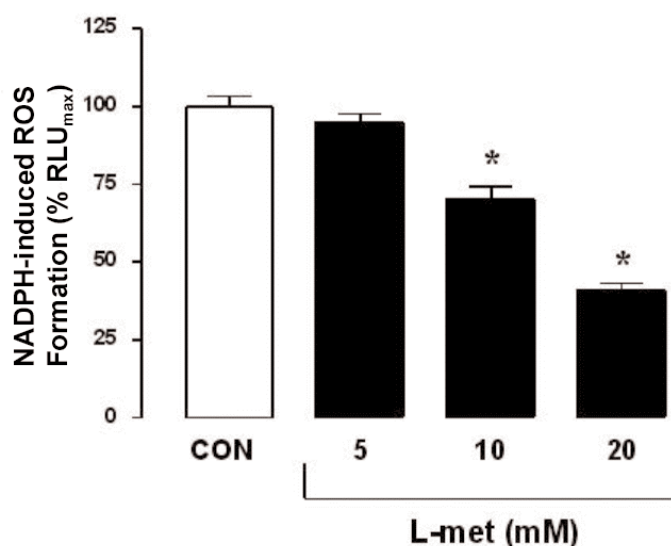
**Figure 1.** L-methionine (L-met) increases heme oxygenase-1 (HO-1) protein expression in a concentration-dependent manner in endothelial cells. The densitometric data are shown as means  $\pm$  SEM of  $n = 5$  independent observations. \* $P < .05$ , treatment vs CON (1-way ANOVA and Bonferroni's multiple comparison test). A representative Western blot analysis is shown in the upper panel.



**Figure 3.** L-methionine (L-met) increases heme oxygenase activity (bilirubin formation) in endothelial cells. Values are means  $\pm$  SEM of  $n = 4$  independent observations. \* $P < .05$ , treatment vs CON (1-way ANOVA and Bonferroni's multiple comparison test).



**Figure 2.** L-methionine (L-met) increases ferritin protein expression in a concentration-dependent manner in endothelial cells. The densitometric data are shown as means  $\pm$  SEM of  $n = 5$  independent observations. \* $P < .05$ , treatment vs CON (1-way ANOVA and Bonferroni's multiple comparison test). A representative Western blot analysis is shown in the upper panel.

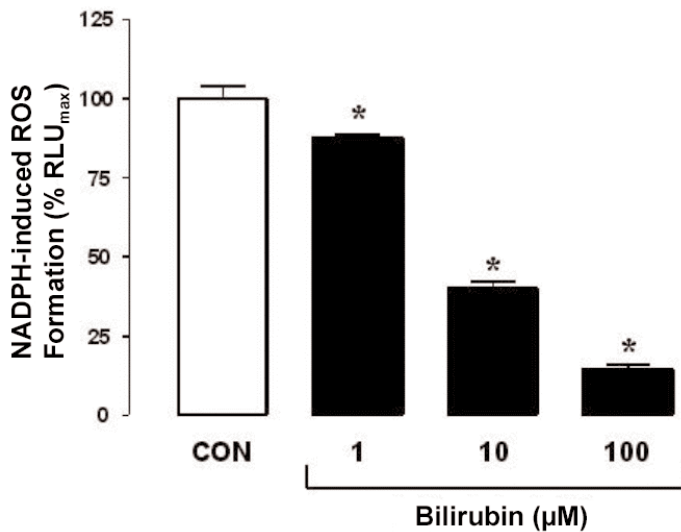


**Figure 4.** Effect of L-methionine (L-met) on NADPH-dependent  $O_2^-$  formation in endothelial cells. Values are means  $\pm$  SEM of  $n = 3$  independent observations. \* $P < .05$ , treatment vs CON (1-way ANOVA and Bonferroni's multiple comparison test).

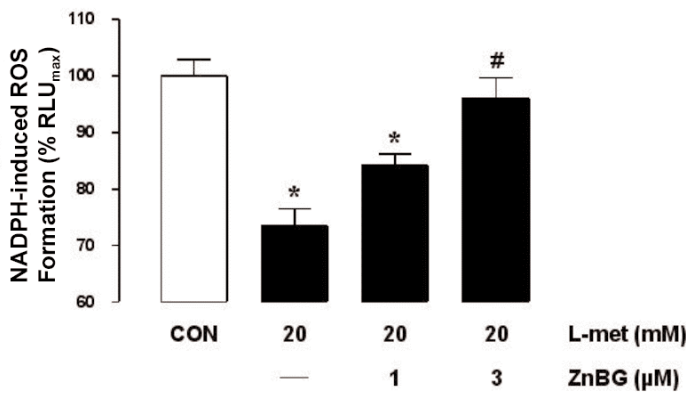
## DISCUSSION AND CONCLUSION

In addition to its role as a precursor in protein synthesis, the amino acid L-methionine has been reported to exert tissue protective actions against free radical-induced injury.<sup>5,6</sup> Moreover, methionine deficiency has been associated with a variety of cardiac and vascular disorders and with carcinogenesis.<sup>26-28</sup> However, the underlying mechanisms that could

explain the antioxidant effects of methionine remain obscure. This study demonstrates that the antioxidant defense protein HO-1 is an intracellular site of action for L-methionine. The amino acid stimulated HO-1 protein expression as well as enzymatic activity in endothelial cells. Pretreatment with L-methionine was associated with protection of endothelial cells from oxidative stress. L-methionine inhibited ROS formation that was elicited by addition of NADPH to the cells following washout of L-methionine, suggesting an indirect antioxidant action of the amino acid rather than direct radical scavenging. Interestingly, the L-arginine antagonist and inhibitor of NO synthase, L-NMMA, abrogated L-methionine

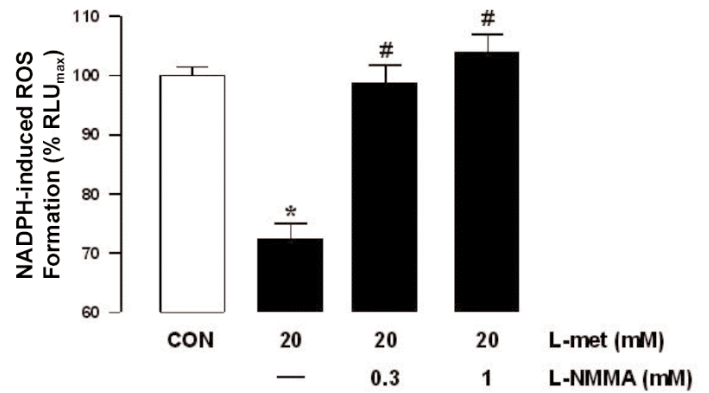


**Figure 5.** Effect of bilirubin on NADPH-dependent  $O_2^-$  formation in endothelial cells. Values are means  $\pm$  SEM of  $n = 3$  independent observations. \* $P < .05$ , treatment vs CON (1-way ANOVA and Bonferroni's multiple comparison test).



**Figure 6.** Effect of the HO inhibitor ZnBG on the antioxidant action of L-methionine (L-met) in endothelial cells. Values are means  $\pm$  SEM of  $n = 5$  independent observations. \* $P < .05$ , treatment vs CON. # $P < .05$ , for treatment with ZnBG and L-methionine vs L-methionine alone (1-way ANOVA and Bonferroni's multiple comparison test).

ine-induced radical scavenging. This finding points to the involvement of endogenous NO, an established HO-1-inducing signaling molecule,<sup>12,29,30</sup> as a mediator of L-methionine-dependent antioxidant actions. The effects of L-methionine were observed at high micromolar and low millimolar concentrations of L-methionine, which are well within the range of plasma levels after methionine loading.<sup>31</sup> Higher concentrations between 5 and 20 mM L-methionine were required to confirm increased HO-1 activity in a cell-free assay of bilirubin. This inevitable loss of sensitivity during the “ex vivo” measurement of specific enzyme activity in a broken cell system has been reported previously and is a result of induced HO-1 not being fully recoverable during the complex preparation of lysate from intact cells that were preexposed to inducing agents.<sup>12,24</sup>



**Figure 7.** Effect of the NO synthase inhibitor L-NMMA on the antioxidant action of L-methionine (L-met) in endothelial cells. Values are means  $\pm$  SEM of  $n = 4$  independent observations. \* $P < .05$ , treatment vs CON. # $P < .05$ , for treatment with L-NMMA and L-methionine vs L-methionine alone (1-way ANOVA and Bonferroni's multiple comparison test).

In recent years, HO-1 has emerged as an important mediator of antioxidant and tissue protective actions. HO-1 antisense and knockout studies as well as clinical investigations of HO-1 promoter polymorphisms have clearly shown that HO-1 assumes a central role in cellular antioxidant defense and, specifically, in vascular protection.<sup>14,32,33</sup> Cytoprotective and anti-inflammatory actions of HO-1 outside the vasculature have also been documented in various tissues including heart, kidney, and neuronal cells.<sup>12,13,15,23</sup> Therefore, it is plausible to assume that the HO-1 induction observed in this study contributes to the antioxidant profile of L-methionine.<sup>5-11</sup> In agreement with this, we found that the antioxidant effect of L-methionine was abolished in the presence of the HO-1 inhibitor ZnBG,<sup>25</sup> suggesting that HO-1 and its enzymatic products are indeed of functional relevance. Moreover, the HO-1 metabolite bilirubin, when added directly to the cells, profoundly reduced NADPH-dependent oxidative stress with ROS formation nearing that of untreated cells. This effect was seen at low micromolar concentrations of bilirubin. These concentrations are in the upper range of the reference interval for bilirubin plasma levels (high-normal bilirubin) that have been associated with reduced atherogenic risk.<sup>34,35</sup> Our findings lend support to the concept of bilirubin as a biologically important antioxidant<sup>36</sup> and to a role of HO-1 in L-methionine-dependent endothelial protection.

Another HO metabolite, CO, has long been considered as being tissue protective solely by its antiplatelet and vasodilatory effects, the latter being of potential benefit also in antagonizing vascular tolerance.<sup>37</sup> However, recent evidence points to direct anti-inflammatory properties of CO,<sup>15</sup> which may complement and support the cytoprotective and antioxidant actions of bilirubin. A third pathway besides CO and bilirubin formation through which HO-1 induction leads to tissue protection is the induction of a secondary antioxidant

protein, ferritin.<sup>38</sup> Ferritin induction not only protects endothelial cells from the damaging effects of iron-catalyzed oxidative injury but has also been observed to affect the progression of atherosclerotic lesions in patients with coronary heart disease.<sup>19,39</sup> In this study, L-methionine produced increases in ferritin protein expression at concentrations that were also shown to result in HO-1 induction. Our observation that methionine is capable of activating iron-dependent pathways is supported by earlier in vivo studies demonstrating increases in hepatic iron and ferritin levels after a methionine-supplemented diet.<sup>40</sup> In light of our investigations, activation of endogenous iron sequestration could be an important mechanism by which L-methionine increases the cellular defense against oxidative injury.

In summary, we have demonstrated for the first time that L-methionine stimulates expression as well as enzymatic activity of the antioxidant defense protein HO-1 in endothelial cells. HO-1 induction was associated with a marked increase in protein expression of a secondary antioxidant protein, ferritin. Increased HO-1 and ferritin expression may contribute to and explain the specific antioxidant actions of L-methionine. Moreover, enhanced expression of these genes in the endothelium and subsequent attenuation of free radical formation might be relevant for the preventive potential of L-methionine and L-methionine-rich diets under conditions of inflammation and oxidative stress.

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